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(57) Abstract

The invention employs liposomes having an outer surface presenting both acidic and basic polar groups, for example phosphatidyl for rapid, high yield generation of thrombin from prothrombin–containing material. The source material for the prothrombin can be the subject to be treated with the product thrombin, providing an autologous thrombin product. Also, a fibrinogen component can be generated from the same blood sample, yielding a fully autologous fibrin sealant when activated with thrombin product from the same blood sample.

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THROMBIN PREPARATION AND PRODUCTS AND FIBRIN SEALANT METHODS EMPLOYING SAME

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the preparation of thrombin material products, and the use of the products inter alia in the production of fibrin sealants. Fibrin sealants are sealant compositions useful for sealing tissue and other medical and surgical purposes, which generally comprise two fluid components having active agents derived from blood plasma namely a polymerizable fibrinogen component and a thrombin activator. When mixed, the components cure to provide a solid film or deposit of fibrin. If the plasma used to prepare one or another components of the sealant is obtained from a subject to be treated with the sealant, the sealant is said to be "autologous". The invention also relates to novel fibrin sealants wherein both the thrombin and the fibrinogen components are autologous and to their preparation .

2. Description of Related Art Including Information Disclosed under 37 CFR 1.97 and 37 CFR 1.98

Customarily, in prior art fibrin sealant compositions, one component contains fibrinogen which becomes insoluble fibrin when polymerized and cross-linked by an activator and the other component comprises an activation mixture which generally includes thrombin. Alternatives to thrombin, such as thrombin analogs and reptile-sourced coagulants, have also been proposed. Depending upon the ingredients present and their strengths, the mixed sealant components will rapidly gel and eventually form a tough insoluble clot. Fibrin sealants are uniquely valuable for certain specialized surgical procedures, for example in otology, and offer advantages in the treatment of a wide range of wound conditions by virtue of their biocompatibility, their fostering of the wound healing process and the fact that no foreign object or material remains on or in the tissue to effect wound closure or hemostasis.

Patient autologous fibrin sealants were developed in the early 1980s as a response to the lack of availability of homologous fibrin sealant in the United States pursuant to FDA regulations proscribing use of sealants prepared from pooled human sera. Known methods of preparing autologous fibrin sealant compositions have focused exclusively on the production of the first component comprising fibrinogen in admixture with Factor XIII. Factor XIII, or fibrin

stabilizing factor, ("FSF"), is a fibrin cross-linking agent which is activated by thrombin in the presence of calcium ions, Ca++. The latter components, thrombin and Ca++ are accordingly usually provided in the second component.

Examples of the preparation of such two-component fibrin sealants may be found inter alia in Epstein U. S. patent number 5,226,877 ("Epstein '877" herein) and Sierra U. S. patent number 5,290,552. Many others exist.

"Epstein '877" teaches a process and apparatus for one-step preparation of fibrinogen adhesive by polyethylene glycol-mediated precipitation from plasma. The Epstein '877 process begins with the withdrawal of whole blood from e.g. autologous donor in the presence of an anticoagulant, such as citrate, and separation of plasma from the red blood cell fraction. Typically, the separation can be effected by centrifugation; a suitable protocol involves spinning at about 2000-5000 g for about 5-10 minutes. After the plasma is separated from the red blood cells, it is treated directly, at ambient temperature, without prior treatment to remove thrombin, with a physiologically acceptable nontoxic precipitant notably polyethylene glycol in a molecular range of 200-8000 supplied as a concentrated solution. For example, an approximately 60% solution of PEG of molecular weight 1000 in saline at pH 7.4, can be added to the plasma in sufficient volume (typically 10% w/v of precipitant solution/final volume) to obtain a resulting concentration of 8-15% w/v PEG in the volume of the final mixture so that the plasma itself is, only slightly diluted. The precipitation of fibrinogen-rich material is complete essentially instantaneously. The Epstein '877 fibrinogen precipitation process can be used as an optional preliminary step in practicing the invention described hereinbelow.

Epstein does not teach preparation of a thrombin component. Epstein '877's autologous fibrinogen adhesive preparation is intended for admixture with known commercial thrombin preparations. Such preparations would commonly have been of bovine origin.

Thrombin is readily available commercially as an off-the-shelf product produced from bovine plasma and is commonly indicated for use as a topical hemostatic agent for treatment of diffuse capillary bleeding. In the past little concern has been focused on the use of bovine thrombin, but in recent times theoretical, psychological and case report problems have emerged.

Thus, recent investigations implicate thrombin in the etiology of various coagulopathies that

occur in some individuals given repeated doses of thrombin. Also, thrombin impurities, for example Factor V, being a relatively high molecular weight protein of bovine origin, can cause an immunological response in humans. A still more serious problem is that antibodies formed against bovine Factor V may in turn react with the host's own Factor V, disrupting normal hemostasis, leading to severe hemorrhage and even death. Theoretically, these problems might be ameliorated by using higher purity thrombin, but the applicant is not aware of enabling teachings regarding such an approach.

A still further drawback arises from recent widespread publicity regarding possible transmission of bovine spongioform encephalopathy (BSE) to humans from beef products. It is believed that this adventitious agent, an infectious protein known as a "prion", may in turn mutate in the host to produce a Cruetzfield-Jakob-like disease state. This is a terminal, incurable neurodegenerative disease and there are currently no known effective treatments for most bovine-sourced products to inactivate or filter the causative prion.

Another approach has been to eliminate the need for thrombin and its associated problems by employing an alternative pathway to produce fibrin. In one such approach, patient autologous plasma is reacted with a snake venom enzyme having some similarities to thrombin, such as batroxobin, under acidic conditions to form a non-aggregating fibrin-1 “prepolymer”. The batroxobin is then removed from the plasma by chromatographic means and the fibrin prepolymer isolated and concentrated. Gelation occurs when a buffer is mixed with the fibrin preparation, bringing the pH back to neutrality. In addition to the difficulties associated with obtaining the batroxobin, the end product sealant may lack adequate strength. The nature of the process is such that no Factor XIII is present in the mixture to strengthen the resultant clot. Furthermore, the fibrinogen is subject to proteolytic cleavage by such snake venoms and the resultant fibrin may lack adequate strength owing to failure to activate all the aggregation sites.

There is accordingly a need for a thrombin product that can be used as an activator in fibrin sealant compositions and which is not subject to these drawbacks. Theoretically, recombinant human thrombin might offer a potential solution but expectations in early 1998 are that such a product is at least five years away. Also the efficacy of such a hypothetical synthetic product, absent natural biological co-factors, is unknown.

There is accordingly still a need for a thrombin product which is an effective activator in a multi-

component fibrin sealant composition, which can be quickly prepared, and which provides a low risk of inducing an immunologic response or disease. Autologous thrombin, thrombin derived from the subject to be treated, meets the requirements of low immunogenicity and infectivity and is a desirable product. However, there are difficulties in obtaining autologous thrombin since the subject's blood plasma is the only possible source of the desired thrombin product. Any method of preparing autologous thrombin should be quick, easily effected in proximity to a surgical environment, without needing special equipment, and capable of producing an effective fibrinogen coagulation activator from a small sample of blood.

Cochrum U. S. patent 5,510,102, and others, teach use of biocompatible polymers such as alginates, poly-L-amino acids, chitosan and chitin in a hemostatic adhesive agent comprising a possibly autologous platelet-rich plasma concentrate having up to 10 times the concentration of normal plasma of , said concentrate containing 5 to 10 times higher concentration of fibrinogen and other plasma proteins than normal plasma. However, Cochrum provides no teaching of a thrombin material that can be mixed with a fibrinogen material to provide a fibrin sealant for surgical use.

Cochrum provides an extensive discussion of the complex blood coagulation cascade that yields wound-protecting clots. As explained by Cochrum, the essential reaction in coagulation of the blood is the enzymatic conversion of the soluble protein fibrinogen into the insoluble protein fibrin by thrombin. Fibrinogen exists in the circulating blood as such. Thrombin is formed from an inactive circulating precursor, prothrombin, as a result of tissue injury, bleeding or blood loss. The activation of prothrombin depends on the presence of calcium ions and on the presence of thromboplastin, factor III, which is released or derived from the damaged tissues, from the disintegrating platelets or from the plasma itself.

Antanavich U.S. patent number 5,585,007 teaches a method of preparing a concentrate for use as a tissue sealant by contacting plasma with a concentrator that absorbs water, electrolytes and small proteins (column 12, lines 29-33) to yield a platelet-rich plasma product. The concentrator is a solid water-absorbent material, for example beads or discs of materials such as polyacrylamide, dextranomer, silica gel or starch. At column 21, lines 7-9, Antanavich teaches that in a further embodiment, the activator can be autologous thrombin and platelets. So far as can be ascertained in the lengthy patent specification, it appears that the only method Antanavich teaches for providing autologous thrombin is to use a needle tip to disrupt platelets, enabling a

single-component concentrate to be applied (as a sealant), without use of bovine thrombin, see column 24, lines 32-35. However, no data is given as to the efficacy of such a single-component concentrate. Again, Antanavich does not appear to provide a thrombin material that can be mixed with a fibrinogen material to provide a fibrin sealant for surgical use.

Toward the objective of a fully autologous fibrin sealant wherein both fibrinogen and thrombin are sourced from the intended patient or other single donor, Hirsh U. S. patent number 5,643,192, teaches that the serum supernatant available after an initial precipitation of fibrinogen from a sample of the intended patient's plasma can be mixed with a calcium chloride solution. Residual fibrin that forms is skimmed out. After at least 15 or more minutes, a dilute (< 20U/ml) thrombin solution is ready. Drawbacks of this process are that it may be unduly time-consuming and that the resultant thrombin solution is weaker and more dilute than is desirable and may yield a sealant which is an ineffective treatment for many of the major conditions for which a fibrin sealant is indicated, notably hemostasis, fluid-tight sealing and microsurgical adhesion.

Cederholm-Williams U.S. patent number 5,795,571 teaches use of a possibly autologous thrombin fraction for treatment of hemostasis, prevention of blood loss, in animals, including humans. Cederholm-Williams lowers the ionic strength and pH of a blood plasma sample from which cells and platelets have been removed to yield a precipitate described as a "euglobulin fraction." The euglobulin fraction contains prothrombin, fibrinogen and many other blood proteins, but is substantially free of antithrombin III a plasma protein which may prevent the conversion of prothrombin to thrombin or may inactivate thrombin. The prothrombin of the euglobulin fraction is then redissolved and converted to thrombin to form the desired thrombin blood fraction, for example by solubilizing the euglobulin fraction in saline, buffered to a neutral pH. However, Cederholm-Williams's method does not provide a separate fibrinogen-containing material to serve as the other component of an autologous fibrin sealant.

Cederholm-Williams provides an extensive bibliography of literature in the field, including a number of references regarding thrombin preparation.

There is accordingly a need for a method of producing thrombin which can quickly and easily provide the thrombin component of an autologous fibrin sealant

SUMMARY OF THE INVENTION

The invention, as claimed, is intended to provide a remedy. It solves the problem of providing an effective thrombin activator for a multi-component fibrin sealant composition, which can be quickly prepared and which, if desired, can be prepared autologously along with an autologous fibrinogen component, from the same blood sample, to provide a fully autologous fibrin sealant.

In one aspect the invention provides a method of preparing a thrombin material suitable for use as an autologous fibrin sealant component, the method comprising exposing prothrombin-containing material to an extended surface area material capable of activating conversion of prothrombin to thrombin in an aqueous medium. The extended surface area material is preferably covered with polar groups of a nature found on the exterior surfaces of activated platelets or endothelial cells. Preferably, the polar groups comprise phosphatidyl groups and basic nitrogen groups, and the extended surface area material comprises phospholipid liposomes. Also, to facilitate the conversion, it is desirable that the aqueous medium comprises factor V, factor X and calcium ions.

Use of such an extended surface area material facilitates the conversion of prothrombin to thrombin, providing an excellent yield of thrombin. It is also desirable, but optional, that the aqueous medium comprise a reduced proportion of antithrombin III relative to prothrombin as compared with the proportions in blood plasma.

It is furthermore preferable that the prothrombin material comprises a precipitate obtained from fibrinogen- and factor XIII-depleted plasma. The fibrinogen- and factor XIII-depleted plasma can be prepared by precipitating fibrinogen and factor XIII from a blood plasma sample from which the cellular components have been removed. The fibrinogen and factor XIII precipitate can be used to prepare the fibrinogen component of a fibrin sealant, and by then using the residual plasma for preparation of the thrombin component, a fully autologous fibrin sealant can be obtained by taking the blood sample from the subject to be treated with fibrin sealant.

In another aspect the invention provides a method of preparing a thrombin material suitable for use as an autologous sealant component, the method comprising:

a) treating a blood plasma sample obtained by removal of cellular and platelet components from a whole blood sample to yield a fibrinogen precipitate containing fibrinogen and factor XIII and to provide fibrinogen- and factor XIII-depleted plasma;

b) treating the fibrinogen- and factor XIII-depleted plasma to yield a prothrombin-containing precipitate; and

c) treating the prothrombin-containing precipitate to yield thrombin.

Preferably, the treatment of the prothrombin-containing precipitate in step c) comprises dissolving the prothrombin-containing precipitate in an aqueous reagent and exposing the dissolved prothrombin-containing material to an extended surface area substrate capable of converting prothrombin to thrombin.

As stated above, the extended surface area material comprises a suspension of phosphatidyl liposomes, preferably with a major proportion of phosphatidyl choline and a minor proportion of phosphatidyl serine.

In a further aspect the invention provides a method of preparing an autologous fibrin composition comprising separate fibrinogen and thrombin components mixable to provide a sealant, the method comprising the steps of:

a) removal of cellular and platelet components from a sample of patient autologous blood to yield a plasma supernatant; and

b) generating a precipitate containing fibrinogen and Factor XIII from the plasma supernatant for reconstitution as the fibrinogen component of the fibrin sealant;

c) treating the plasma supernatant from step b) to yield a prothrombin-containing precipitate; and

d) separating the prothrombin-containing precipitate from step c) and dissolving the prothrombin-containing precipitate in a sufficient quantity for solution of a tissue-compatible aqueous activation reagent comprising:

i) a sufficient quantity of calcium chloride, or an equivalent thereof, to be effective in the tissue sealant; and

ii) an effective quantity of a dispersed, tissue-compatible prothrombin-converting particulate substrate;

to provide a thrombin-containing component.

The particulate substrate can comprise phosphatidyl liposomes having a major proportion of phosphatidyl choline and a minor proportion of phosphatidyl serine while the treatment of step c) comprises reducing the ionic strength and the pH of the solution to the isoelectric point of

prothrombin.

The invention also comprises thrombin material produced by any of the methods of the invention and autologous fibrin sealant compositions produced by an inventive method.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

One or more embodiments of the invention and of making and using the invention, as well as the best mode contemplated of carrying out the invention, are described in detail below, by way of example, with reference to the accompanying drawings, in which:-

Figure 1 is a block flow diagram of one method of preparing thrombin, according to the invention; and

Figure 2 is a block flow diagram of a method of preparing thrombin, according to a preferred embodiment of the invention, which embodiment is described more fully hereinbelow in connection with Example 2.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Referring now to Figure 1, the invention provides, in one embodiment, a method of preparing an optionally autologous thrombin composition suitable for use in a fibrin sealant comprising the steps of:

a) collecting a suitable amount of patient autologous blood, for example about 100 ml or less, in a citrate solution, or equivalent aqueous medium;

b) removing cellular and platelet components from the solution, for example by centrifugation or filtration, to yield a plasma supernatant;

c) generating a precipitate containing fibrinogen and Factor XIII from the plasma supernatant for reconstitution as a fibrinogen component of the fibrin sealant;

d) treating the serum supernatant from step c) to yield a prothrombin-containing precipitate;

e) separating the prothrombin-containing precipitate from step d), for example by centrifugation or filtration;

f) dissolving the prothrombin-containing precipitate from step e) in a sufficient quantity for solution of a tissue-compatible aqueous activation reagent comprising:

i) a sufficient quantity of calcium chloride, or an equivalent thereof, to be effective in the tissue sealant; and

ii) an effective quantity of a dispersed, tissue-compatible prothrombin-converting particulate substrate;

to provide the desired thrombin-containing component.

Preferably, the prothrombin-converting particulate substrate comprises liposomes having bipolar external moieties, for example, such as provided by phospholipids, for example lecithin-based phospholipids. Preferably also, the liposome particles are relatively large in size, being of the order of 0.1 to 5 mm in diameter, more preferably about 1 or 2 mm. Alternatively, the particulate substrate can comprise platelets collected and isolated from the patient's blood or from a screened donor, for example a close relative. Alternatively rather than particles a lipid emulsion may be used. A suitable quantity of substrate particles is from about 1 to about 500 mg, preferably from about 5 to about 100 mg and more preferably about 10 to about 50 mg per 100 ml. of original blood volume. The substrate material can be incorporated in from about 0.5 to about 100ml, preferably about 1 to about 20 ml, and more preferably about 2 to about 20 ml of saline, optionally with calcium chloride.

Preferably, the pH is also raised back to about 7 and extraneous fibrin is removed as the Factor 11 prothrombin converts to thrombin precipitating residual dissolved fibrinogen as fibrin.

After a short time, less than 10 minutes, for example about 3 minutes, the thrombin-containing component is ready for use as the second, activation component of the fibrin sealant. The first sealant component is preferably the product of step c), although, as will be understood by those skilled in the art, the novel thrombin product of step f) could be used with other non-autologous fibrinogen components, or for other purposes, if desired.

The thrombin-containing component can be obtained as a concentrated thrombin solution with a yield of about 2 ml from a 100 ml blood sample. In a preferred embodiment the concentration is sufficient for a gelation time of about 5 seconds when the thrombin-containing component is mixed in a 1:1 volume ratio with the fibrinogen component 1 of step c). The invention thus provides an autologous thrombin fibrin sealant component capable of effecting gelling in less than one minute, preferably less than 30 seconds and still more preferably, when desired, in about 10 seconds or less.

The entire process of producing the two sealant components can be completed in about 20-30 minutes. The majority of the processing time is taken up with centrifugation rather than attention-requiring precipitation and activation steps. This short time and the lack of

requirements for special equipment suit the process to be carried out in conjunction with an surgical theater environment where centrifuges are usually available.

The precipitate resulting from step c) can be isolated by filtration or centrifugation to provide the first component of a fibrin sealant, yielding about 2 ml. A variety of methods of performing step c) is known to the art and may be used, some of which are described in the literature cited herein, for example, polyethylene glycol ("PEG")-citrate precipitation, cryoprecipitation, ammonium sulfate precipitation and ethanol precipitation.

The prothombin precipitating treatment of step d) can be effected with a mildly acidic aqueous medium, or other precipitating agent. for example a buffered polyethylene glycol solution. Preferably, the aqueous diluent used in step d), which can be water, is used in a proportion of from about 1:1 to about 20:1 to the quantity of blood collected, more preferably from about 2:1 to about 10:1, and still more preferably, from about 3:1 to about 5:1. The acidity is preferably in a pH range of about 4 to about 6, more preferably about 5. Preferably also antithrombin III is inactivated, for example by such acidification.

In step e) the prothrombin-containing precipitate is preferably isolated and concentrated and the supernatant can be discarded.

In a preferred embodiment, in step f) and the aqueous component of the reagent comprises isotonic saline, calcium chloride and the phospholipid liposomes.

It will be understood that the above-mentioned quantities are relative to an in initial blood sample aliquot of about 100 ml, and are simply indicative of relative proportions that may be used, depending upon the size of the aliquot.

While not being bound by any particular theory, the invention can be further described in the following terms. In step c), fibrinogen and Factor XIII are precipitated out of the collected plasma while the remaining coagulation factors, specifically II, V and X are subsequently precipitated in step d), for example by altering the pH of the serum supernatant, so that fibrinogen and factor FIII attain their isoelectric points. Selectivity for these particular proteins can be enhanced by reducing the ionic strength of the serum by dilution with purified water, for example to from two to ten times the original blood sample volume, preferably about four or five times.

In order to convert the zymogen Factor II, prothrombin, into its active form, thrombin, several components can be employed, specifically, Factor V, Factor X, Ca^{++} and the substrate particles, preferably phospholipid liposomes. The presence or absence of any of these components can directly impact the rate of thrombin production. Factors V and X become activated when calcium ion as a cofactor becomes available. These three entities are believed to form a non-covalently bound complex in which Factor 11 undergoes proteolytic cleavage to form thrombin.

The present inventor has discovered that the use of a synthetic activator presenting a phospholipid surface for this complex to bind to can dramatically increase the rate of thrombin production compared with what would otherwise be obtainable, for example by several orders of magnitude. A preferred composition comprises a major proportion of phosphatidyl choline (PC) and a minor proportion of phosphatidyl serine (PS) in a ratio of from about 1:1 to about 5:1, preferably about 7:3. Such a composition is believed to provide an external surface with similarities to that of activated platelets or endothelial cells. While, as has been conventional phospholipid of this composition can be obtained from brain extracts of rabbit, sheep or bovine origin it is preferred for the purposes of this invention, that, liposomes preferably having a mean diameter of at least 1 mm are produced from a synthetically derived lipid preparation (e.g. "Synthetic Phospholipid Blend", Avanti Polar-Lipids, Inc. Alabaster. AL), "PCPS" hereinafter. This composition apparently closely approximates platelets and endothelial cells in composition and physical configuration and may contribute to reaction efficiency.

Liposomes or "lipid bodies", sometimes called "vesicles" are structures formed spontaneously by polar lipid molecules, or amphiphilic molecules, each having a polar head and a long hydrophobic tail, e.g. phospholipids such as lecithin. Structurally, liposomes comprise an outer shell of one or more membrane-like, bi-layers of the molecules arranged concentrically around a hollow interior, or "vacuole" which can serve as a storage compartment for active agents. In the outer layer the polar heads of the molecules are oriented outwardly of the liposome, while the hydrophobic tail, e.g. palmitic or stearic acid, depends inwardly. Lecithin liposomes composed primarily of phosphatidyl choline can be expected to be coated or covered, or to present an outer layer which comprises an array of dipoles. Thus lecithin molecules have polar heads which align to the outside of the liposome particle, and each has twin hydrophobic, lipophilic fatty acid tails which align inwardly. The polar character of the head of each lecithin molecule has the nature of a dipole with a positive charge located in the vicinity of the molecule's distal quaternary nitrogen atom, and a negative charge in the vicinity of its neighboring phosphatidyl

group. Phosphatidyl serine has a dipolar head provided by amino and phosphatidyl groups and a mixture of phosphatidyl choline and phosphatidyl serine will provide a surface of quaternary nitrogen, amino and phosphatidyl groups.

Many suitable materials for and methods of preparation of liposomes are known to the art, see for example Unger et al. ("Unger") U.S. Patents 5,469,854 and 5,580,575 and can be used to provide the extended surface area material of the invention. Alternatively, the extended surface area material can comprise a continuous substrate, for example a column of material such as SEPHADEX (trademark). Preferably, the extended surface area material comprises a surface layer of polar groups providing dipoles, namely pairs of positively and negatively charged groups, more preferably basic nitrogen groups or other biologically available positively charged groups, for example chelated or otherwise complexed metallic groups such as ferrous or ferric and phosphatidyl or carboxyl or other negatively charged groups.

Example 1: (Comparative Example) A Known Thrombin Production Method

In a beaker, 100 ml of citrated bovine plasma are diluted with 900 ml of deionized water. The pH is adjusted to 5.3 with 2% acetic acid. The solution is centrifuged for 10 minutes at 2000 X g at 4°C. The supernatant is decanted and discarded. The pellets are collected and resuspended in 25 ml of saline and 3 ml of 0.25 M $CaCl_2$. The pH is raised to 7 with 2% Na_2CO_3. The mixture is incubated for 2 hours and the precipitating fibrin skimmed off and discarded. Acetone (25 ml) is added and the mixture centrifuged for 10 minutes at 2000 X g at 4°C. Saline (25 ml) is added to extract the thrombin and the precipitate discarded. When mixed with concentrated fibrinogen-FX111, no gelation was noted. When the above method was repeated with serum from PEG-citrate precipitated fibrinogen (using the Epstein '877 method), a gelation time of 30 seconds was observed. The resultant gel was of a friable consistency.

Example 2: Thrombin Production Method with PEG-Precipitated Fibrinogen Serum

Referring now to Figure 2, two 50 cc centrifuge tubes containing 33 ml each of citrated plasma are mixed with 17 ml each of PEG-citrate precipitation solution. The tubes are centrifuged for 10 minutes at 3600 rpm at 4 °C and the serum supernatant decanted into a beaker. The fibrinogen-FXIII precipitate is collected and diluted 1:1 v/v with saline. To the serum supernatant, 400 mL of deionized water are added and the pH adjusted to 5.3 with 2% acetic acid. The solution is centrifuged for 10 minutes at 2000 X g, and the supernatant decanted and discarded. The pellets are collected and dissolved in 5 mL of a suspension consisting of isotonic

saline, 30 mM CaCI2 and 20 mg of PCPS (7:3) liposomes. This conversion reagent was prepared by adding 5 ml of the saline-$CaCl_2$ solution to a vial containing 20 mg of lyophilized PCPS lipid and a magnetic stir bar and vortexing intermittently over the course of at least 15 minutes, and placed on a stir plate between vortexing. Fibrin formation from unprecipitated fibrinogen occurs in about 90 seconds upon addition of the liposome suspension. The fibrin is removed and no further formation is observed after approximately 4 minutes. To a glass culture tube, 1 mL of the PEG-precipitated fibrinogen-FXIII solution is added to 1 mL of the thrombin solution. Gelation of the mixture was observed within 5 seconds. This experiment demonstrates one method of producing both fibrin sealant components from the same relatively small plasma sample within a short time (25 minutes).

Example 3: Thrombin Production Method with Cryosupernatant (Serum)

100 mL of citrated bovine plasma are frozen at -2O°C for 24 hours, then slowly thawed for 24 hours at 4°C. The plasma-precipitate is centrifuged for 10 minutes at 3600 rpm also at 4 °C and the serum supernatant decanted. The cryoprecipitate is collected and set aside for later use as the fibrinogen component of a fibrin sealant. The serum supernatant (ca. 97 mL) is diluted with 400 mL deionized water and the pH reduced to 5.3 with 2% acetic acid. The same methodology as described in Example 2 is used to prepare the thrombin solution. Onset of fibrin formation in the thrombin solution occurs in 50 seconds. A gelation time of 5 seconds of a 1:1 v/v mixture of the thrombin and the cryoprecipitate was obtained. This experiment illustrates that the improved thrombin production method is compatible with fibrinogen preparation methods other than PEG-citrate.

The method of the invention may be scaled down in terms of dilution volume reduction, however, the activation time of the thrombin becomes increased with a decreasing water/serum ratio. The method may be performed using a simple arrangement of vessels such as syringes, blood handling bags, stopcocks and/or extension tubes. Parts of, or the entire assembly may be placed in a clinical centrifuge. Alternatively, the entire procedure may be done in an automated machine with a drop-in cartridge containing the disposable blood contacting surfaces and solutions specific for that particular case.

The residual fibrinogen in the thrombin solution may be eliminated by either improving the efficiency of the fibrinogen precipitation reaction step or by using a means of immobilizing the resultant fibrin in the thrombin conversion vessel. These methods include for example: coating

biocompatible or biodegradable particulate or microspheres with PCPS, then centrifuging out the fibrin bound to the particulate. This may be accomplished by having the thrombin reaction occurring under agitation to prevent gel formation. The microspheres or particulate may be magnetized, then when a magnetic field is applied, the fibrin-bound material is separated from the thrombin supernatant. The surface of the reaction vessel may be coated with the PCPS lipid and the vessel centrifuged or spun longitudinally to separate the fibrin. An insert with vanes coated with the lipid could be placed in the reaction vessel to increase further the efficacy and efficiency of the fibrin removal.

As an alternative product concept, a patient autologous or screened single donor thrombin solution for use as a topical hemostat could be conveniently prepared with the patient's own blood in the operating room or blood bank. Donor serum may be used as well. Preparation of this could be done in a closed system. Such a product could be useful in patients known to be sensitive to bovine products or with coagulopathy from previous dosages of bovine thrombin.

INDUSTRIAL APPLICABILITY

The present invention is particularly suitable for application in the health industry providing new means of treating wounds and other tissue problems.

While some illustrative embodiments of the invention have been described above, it is, of course, understood that various modifications and equivalents of the described embodiments will be apparent to those of ordinary skill in the art. Some equivalents will be readily recognized by those of ordinary skill while others may require no more than routine experimentation. Such modifications and equivalents are within the spirit and scope of the invention, which is limited and defined only by the appended claims.

Claims:

1. A method of preparing a thrombin material suitable for use as an autologous fibrin sealant component, the method being **characterized by** comprising exposing prothrombin-containing material to an extended surface area material capable of activating conversion of prothrombin to thrombin in an aqueous medium.

2. A method according to claim 1 **characterized in that** the extended surface area material is covered with polar groups of a nature found on the exterior surfaces of activated platelets or endothelial cells.

3. A method according to claim 1 **characterized in that** the polar groups comprise phosphatidyl groups and basic nitrogen groups.

4. A method according to claim 1, 2 or 3 **characterized in that** the extended surface area material comprises phospholipid liposomes.

5. A method according to claim 1 **characterized in that** the aqueous medium comprises factor V, factor X and calcium ions.

6. A method according to claim 1 **characterized in that** the aqueous medium comprises a reduced proportion of antithrombin III relative to prothrombin as compared with the proportions in blood plasma.

7. A method according to claim 1 **characterized in that** the prothrombin material comprises a precipitate obtained from fibrinogen- and factor XIII-depleted plasma.

8. A method of preparing a thrombin material suitable for use as an autologous sealant component, the method comprising:

a) treating a blood plasma sample obtained by removal of cellular and platelet components from a whole blood sample to yield a fibrinogen precipitate containing fibrinogen and factor XIII and to provide fibrinogen- and factor XIII-depleted plasma;

and being **characterized by:**

b) treating the fibrinogen- and factor XIII-depleted plasma to yield a prothrombin-

containing precipitate; and

c) treating the prothrombin-containing precipitate to yield thrombin.

9. A method according to claim 8 **characterized in that** the treatment of the prothrombin-containing precipitate in step c) comprises dissolving the prothrombin-containing precipitate in an aqueous reagent and exposing the dissolved prothrombin-containing material to an extended surface area substrate capable of converting prothrombin to thrombin.

10. A method according to claim 9 **characterized in that** the extended surface area material comprises a suspension of phosphatidyl liposomes.

11. A method according to claim 10 **characterized in that** the phosphatidyl liposomes comprise a major proportion of phosphatidyl choline and a minor proportion of phosphatidyl serine.

12. A method of preparing an optionally autologous fibrin composition comprising separate fibrinogen and thrombin components mixable to provide a sealant, the method comprising the steps of:

a) removal of cellular and platelet components from a sample of patient autologous blood to yield a plasma supernatant; and

b) generating a precipitate containing fibrinogen and Factor XIII from the plasma supernatant for reconstitution as the fibrinogen component of the fibrin sealant;

characterized by

c) treating the plasma supernatant from step b) to yield a prothrombin-containing precipitate; and

d) separating the prothrombin-containing precipitate from step c) and dissolving the prothrombin-containing precipitate in a sufficient quantity for solution of a tissue-compatible aqueous activation reagent comprising:

i) a sufficient quantity of calcium chloride, or an equivalent thereof, to be effective in the tissue sealant; and

ii) an effective quantity of a dispersed, tissue-compatible prothrombin-converting particulate substrate;

to provide a thrombin-containing component.

13. A method according to claim 12 **characterized in that** the particulate substrate comprises phosphatidyl liposomes having a major proportion of phosphatidyl choline and a minor proportion of phosphatidyl serine.

14. A method according to claim 13 **characterized in that** the treatment of step c) comprises reducing the ionic strength and the pH of the solution to the isoelectric point of prothrombin.

15. Thrombin material **characterized by** being produced by the method of claim 1.

16. Thrombin material **characterized by** being produced by the method of claim 8.

17. An autologous fibrin sealant composition **characterized by** being produced by the method of claim 12.

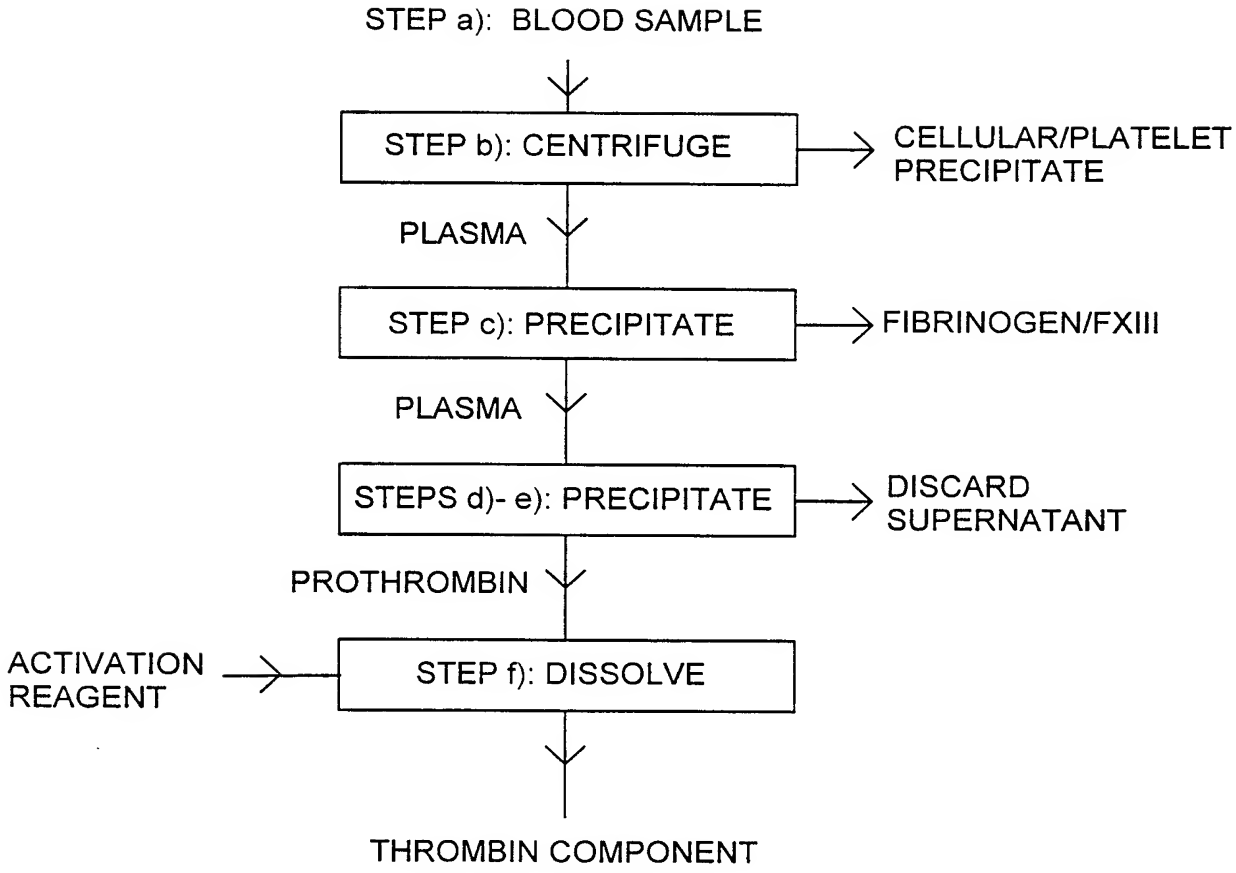
STEP a): BLOOD SAMPLE
STEP b): CENTRIFUGE
CELLULAR/PLATELET PRECIPITATE
PLASMA
STEP c): PRECIPITATE
FIBRINOGEN/FXIII
PLASMA
STEPS d)- e): PRECIPITATE
DISCARD SUPERNATANT
PROTHROMBIN
ACTIVATION REAGENT
STEP f): DISSOLVE
THROMBIN COMPONENT

Figure 1

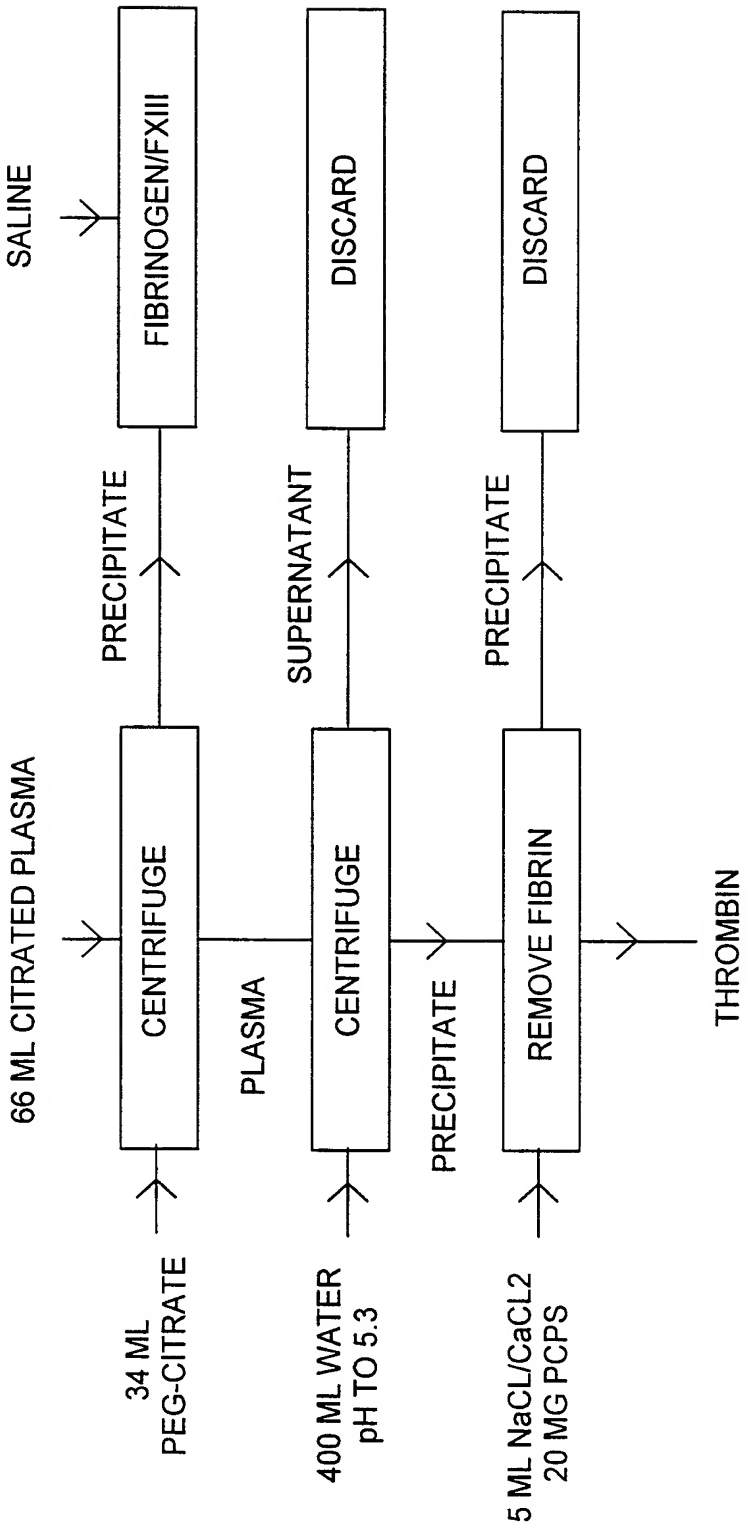
66 ML CITRATED PLASMA
34 ML PEG-CITRATE
CENTRIFUGE
PRECIPITATE
SALINE
FIBRINOGEN/FXIII
PLASMA
400 ML WATER pH TO 5.3
CENTRIFUGE
SUPERNATANT
DISCARD
PRECIPITATE
5 ML NaCL/CaCL2 20 MG PCPS
REMOVE FIBRIN
DISCARD
THROMBIN

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05034

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/14, 35/16
US CL : 424/529, 530, 532, 94.64
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : A61K 35/14, 35/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, CAPLUS, MEDLINE, APS
search terms: thrombin, liposom?, membran?, phosphatidyl, fibrin, glue, seal?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	JONES. M.E. et al. Comparison of the Abilities of Synthetic and Platelet-Derived Membranes to Enhance Thrombin Formation. Thrombosis Research. 1985. Vol. 39. pages 711-724, see the entire document.	1-7, 15 ---------- 8-14, 16-17
X -- Y	SMEETS. E.F. et al. Contribution of Different Phospholipid Classes to the Prothrombin Converting Capacity of Sonicated Lipid Vesicles. Thrombosis Research. 1996. Vol. 81. No. 4. pages 419-426, see the entire document.	1-7, 15 ---------- 8-14, 16-17
X -- Y	KUNG. C. et al. A Membrane-Mediated Catalytic Event in Prothrombin Activation. Journal of Biological Chemistry. October 14, 1994. Vol. 269. No. 41. pages 25838-25848, see the entire document.	1-7, 15 ---------- 8-14, 16-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JUNE 1999	Date of mailing of the international search report 12 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEAN C. WITZ Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05034

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,502,034 A (**HOLLY** et al.) 26 March 1996, col. 5, lines 29-48.	1-7, 15 --------- 8-14, 16-17
Y	US 5,330,974 A (**PINES** et al.) 19 July 1994, see the entire document.	8-14, 16-17
Y	US 5,405,607 A (**EPSTEIN**) 11 April 1995, see the entire document.	8-14, 16-17
Y	US 5,219,995 A (**HERRING** et al.) 15 June 1993, see the entire document.	1-17
Y	US 5,266,462 A (**HEMKER** et al.) 30 November 1993, see the entire document.	1-17